Knockdown of the Yes-associated Protein 1 pathway provides a basis for targeted therapy to treat infantile hemangioma

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Dianna M. Nord[1]

School of Biology
Georgia Institute of Technology
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Approved by:

Dr. Jung Choi
School of Biology
Georgia Institute of Technology

Dr. Frank Stewart
School of Biology
Georgia Institute of Biology

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Acknowledgements
Michael Bonner[2,3], Jack L. Arbiser[2,3]

Affiliations
[1] Georgia Institute of Technology, School of Biology, Atlanta, GA.
[2] Department of Dermatology, Emory School of Medicine, and Winship Cancer Institute, Atlanta, GA.
[3] Dermatology Veterans Affairs Medical Center, Decatur, GA.

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Abstract

Hemangioma is a type of tumor commonly found in infants that is characterized by heavy vascularization and a disfiguring appearance. Hemangioma, though benign, can sometimes proliferate and be threatening to infants. Current treatments for infantile hemangioma include surgical removal as well as the use of topical and oral medication. However, current therapies are often ineffective at treating lesions and are commonly accompanied by dangerous side effects, creating the need for a new, safer treatment. This study targets the Yes-Associated Protein-1 (YAP-1), which has been described as an oncogene, by use of an interfering RNA technique in attempts to mediate tumor growth and progression. Western blotting of treatment and control BEND3 murine cells reveals that YAP-1 is knocked-down in treatment groups which have been infected with shYAP-1 siRNA genes. By successfully knocking down the YAP-1 protein, the potential for developing a novel targeted therapy for infantile hemangioma has been established.

Introduction

Hemangioma is a type of benign vascular tumor that affects approximately 5% of all infants in the world. Hemangioma typically proliferates in infants within the first few weeks of life and is characterized by heavy vascularization and a disfiguring cosmetic appearance. While most hemangioma cases resolve on their own within a few months to two years, they sometimes require medical attention to mediate the severe consequences associated with their formation.[1] Hemangioma proliferation can lead to tissue damage, cosmetic disfigurement, and blockage of normal flow of blood, sometimes to the point of impairing organ function. Severe, untreated hemangioma cases have been shown to continually proliferate and if untreated, can threaten development, vision, and even an infant's life. [4]

There are a number of treatment options available for infantile hemangioma cases that demand therapy. One option is to remove the tumor via surgery and to reconstruct any scarred tissue. However, surgery is not commonly viewed as an ideal treatment for infants and a less invasive treatment option is often preferred. Common treatments in addition to surgery include the use of topical, intralesional, or oral medications such as glucocorticosteroids and propranolol. However, each of these treatment options have been shown to be associated with ineffective results and unfavorable side effects. Though multiple treatment options exist for infantile hemangioma, an ideal therapy free of harmful side effects has yet to be identified.

Literature Review

The most common therapy used to treat infantile hemangioma over the span of the last 40 years has been the use of corticosteroids and glucocorticosteroids. These steroids are typically injected directly into hemangioma tumors in patients. Glucocorticosteroids have been shown to clinically reduce hemangioma tumors and heal infected skin and because of that, they are still widely used today. However, use of corticosteroids to treat hemangiomas have been linked to poor side effects amongst infants. One study that speaks against this therapy showed that the use of corticosteroids causes irritability, fussiness, gastrointestinal problems, and insomnia in many patients and leads to more serious health problems in some such as hypertension and hypothalamic-pituitary-adrenal suppression.[3] Recent studies continue to show that steroid administration can be successful, but can lead to serious issues with the cardiovascular system, gastrointestinal tract, and other systems of the body.

Recently, a new and popular therapy for hemangioma has been developed: the use of propranolol, a type of beta-blocker. Propranolol has been shown in multiple studies to suppress not

only the growth and proliferation of hemangioma, but has also been shown to remedy existing tumors. Many studies link the administration of oral and topical propranolol to successful resolution of hemangioma lesions. Moreover, the propranolol compound has been conveniently manufactured into oral and topical supplements which can be administered fairly easily, perhaps contributing to their popularity amongst physicians. Treating a legion via a supplement is easier than prescribing surgical removal and can potentially be just as successful.

Administering propranolol has been associated with negative side effects in multiple studies. In a recent array study in which many patients were analyzed following oral propranolol administration, cases of tachycardia and stunted growth characterized by the infant's inability to gain weight were reported. Additionally, not all hemangioma lesions were successfully treated with this administration.[5] In addition to poor success rates and side effects, the use of beta blockers has been shown to be effective but accompanied by significant rebound in many patients, meaning that the tumors reappear after treatments conclude and require further, more invasive treatments. A recent study showed 6% of infantile patients had complete rebounding of their hemangiomas within an average of 5.3 months after successful treatment with oral propranolol.[7] Another study amongst Chinese infants showed a complete rebound rate of 17% with the average rebound time being just 9.5 months after conclusion of propranolol treatment.[2] Though successful suppression has been linked to propranolol use, numerous studies have made it clear that there should be a more reliable treatment for these hemangioma cases.

The side effects and risk factors associated with current hemangioma treatments make use of these therapies unpopular with infants. Therefore, it is important for new treatments to be investigated in attempts to better treat infantile hemangioma. While much has been done to examine potential compounds that suppress and heal hemangiomas, little research has been conducted to examine the potential effects of manipulating the genetic makeup of the tumor. There have been many successful studies in the realm of tumor research which have used the manipulation of proteins, genes, and signal transduction pathways to successfully suppress growth and formation in a variety of invasive and non-invasive tumors.[6] Applying these principals to hemangioma could make for a novel therapy in which manipulation of the signal transduction pathways could potentially suppress these benign tumors.

This experiment focuses on the Yes-Associated Protein 1 (YAP-1). YAP-1 is a downstream component of the Hippo signaling pathway that interacts with and modulates various other transcription factors.[9] YAP-1 has been described as an oncogene due to its abilities to promote cell proliferation and transformation, especially in carcinomas. High YAP-1 expression levels have been linked to proliferation and poor survival in malignancies.[10] This evidence supports the idea that its manipulation could have an effect on hemangioma development. RNAi techniques serve to knockdown a targeted protein by preventing transcription. This is done by using an siRNA (short interfering RNA) molecule that is introduced into a cell and binds to a targeted mRNA fragment that codes for the target protein to be manipulated.[11] RNAi technology has been shown to be a successful way to knockdown protein expression and can allow therapies to manipulate proteins. Here, we tested the potential of using RNAi to inhibit YAP-1 expression in mouse hemangioma cells.

Materials & Methods

BEND3 cell model. The bEnd.3 [BEND3] (ATCC® CRL-2299TM) murine cell model of hemangioma was used in this experiment. The cells were cultured and maintained in high-glucose vascular epithelial growth factory (HGVEGF) media and stored in a 37°C incubator.

Control and treatment BEND3 cells. The control BEND3 cells were maintained as normal in HGVEGF media and stored in a 37°C incubator for the entire duration of the experiment. The treatment BEND3 cells were used for RNAi manipulation of YAP-1 expression.

Infection of BEND3 treatment cells with the shYAP-1 gene using Lentiviral Transduction Particles. To perform the interfering RNA technique, shYAP-1 lentiviral transduction particles were used (Sigma-Aldrich product #SHCLNV-NM_009534). These particles were tagged with puromycin resistance and green fluorescent protein (GFP). By using a slight modification of the Sigma-Aldrich protocol titled "Lentiviral Titer by Limiting Dilution", the cells were infected with the shYAP-1 gene, which prevents transcription of the mRNA coding for YAP-1.

Preparation of Lentivral Particle dilutions
Two 6-welled plates were used to carry out the iRNA technique with approximately 2.0×10^5 BEND3 cells taken from the control culture placed into each well. The cells were permitted to grow as normal in the plates for approximately 24 hours in an incubator at 37C. Media containing hexadimethrine bromide was infected with various quantities of lentiviral particles to form dilutions. Vials containing 1mL of media were prepared with 10µL, 15 µL, and 20 µL of lentiviral particles, creating particle dilutions of $1x10^{-5}$, $7.5x10^{-4}$ and $5.0x10^{-4}$. Hexadimethrine bromide was used to ensure transduction of the BEND3 cells.

Infection of cells with lentiviral particle dilutions
Each of the three dilutions was used to infect cells in three separate wells. 1mL of the prepared media with hexadimethrine bromide and the selected dilution was added to each well. For approximately 4 days after this infection, the wells were periodically treated with small amounts of puromycin to control for cells that did not properly undergo transduction by the lentivirus.

Evaluating colony growth
After approximately four days of treatment with puromycin, the wells were examined for growth. The goal at this step was to culture cells from the wells that grew distinct colonies yet were not overgrown. Cells were taken from each well of the optimal dilution to inoculate new flasks, which were named BEND3 YAP-1,1, BEND3 YAP-1,2, and BEND3 YAP-1,3 to correspond to their parent well. Shortly after this step, the BEND3 YAP-1,2 flask was discarded because of insufficient growth. The BEND3 YAP-1,1 and BEND3 YAP-1,3 cells were observed under a fluorescent microscope. The presence of GFP inside the cells indicates successful transduction of the cells by the lentivirus.

Confirmation of protein levels using Western Blotting. A western blot was performed with the control and treatment BEND3 cells in order to examine expression levels of YAP-1. Control BEND3 cells as well as BEND3 YAP-1,1 and BEND3 YAP-1,3 cells were each loaded into one well of a gel in wells 2-4. To ensure results were obtained, the protocol was replicated with wells 7-9 and the cells were loaded a second time in the gel. After running the gel, YAP-1 as well as other relevant proteins were identified on the blot. The blot was treated with a primary antibody as well as an anti-YAP-1 rabbit antibody. The blot was exposed using a Bio-Rad Chemiluminescence imaging system and a luminol substrate, which works by binding to the secondary antibody on the blot. The proteins selected for examination are proteins that are known to be commonly relevant to the development of cancer.

Results

The wells infected with the $5x10^{-4}$ dilution of lentiviral particle cells showed optimal growth, with distinct colonies that were not overgrown. The wells infected with the $7.5x10^{-4}$ and $1x10^{-5}$ dilutions showed too much growth. The $5x10^{-4}$ dilution wells were used as the treatment group of cells. The cells in the second well were discarded because of improper growth. A small colony of cells was taken from the first well and used to inoculate a new flask of cells named BEND3 YAP-1, 1. Likewise, a small colony of cells was taken from the third well and was named BEND3 YAP-1, 3. These cells were maintained in 25mL flasks in HGVEGF media. Both BEND3 YAP-1,1 and BEND3 YAP-1,3 were examined under a fluorescent microscope. GFP appeared inside the cells from both colonies, indicating that the lentiviral particles successfully delivered the shYAP-1 gene to the cells.

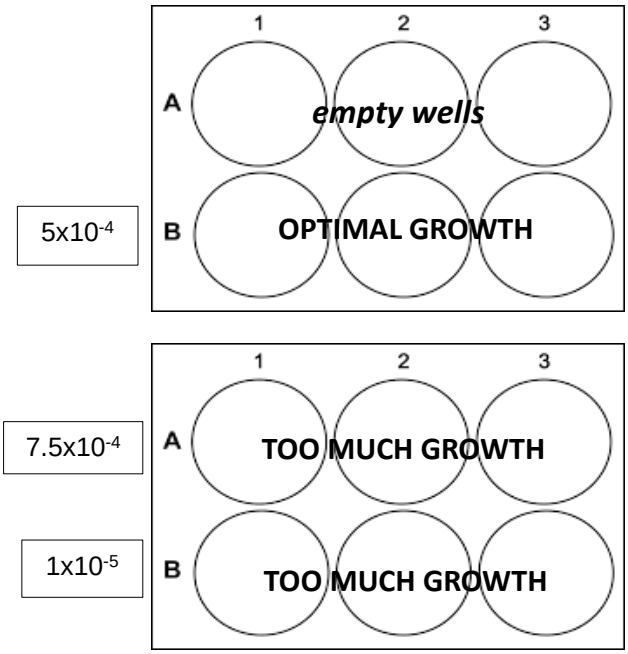

Figure 1: Illustration of 6-welled plate growth 4 days after lentiviral infection.

Western blotting of the treatment and control BEND3 cells reveals there is a difference in protein expression levels when cells have been infected with lentiviral particles containing the shYAP-1 gene. The blot shows that levels of YAP-1 in the treatment and control groups vary. Both groups of BEND3 cells that contain the shYAP-1 gene (BEND3 YAP-1, 1 and BEND3 YAP-1, 3) have down-regulated YAP-1 expression compared to the BEND3 cells that have not been manipulated. In addition, other relevant proteins were examined on this blot. The blot reveals that other proteins are effected by the YAP-1 knockdown. P-MAPK 44/42 appears to be down-regulated in the treatment cells while p-AKT S473 appears to be up-regulated.

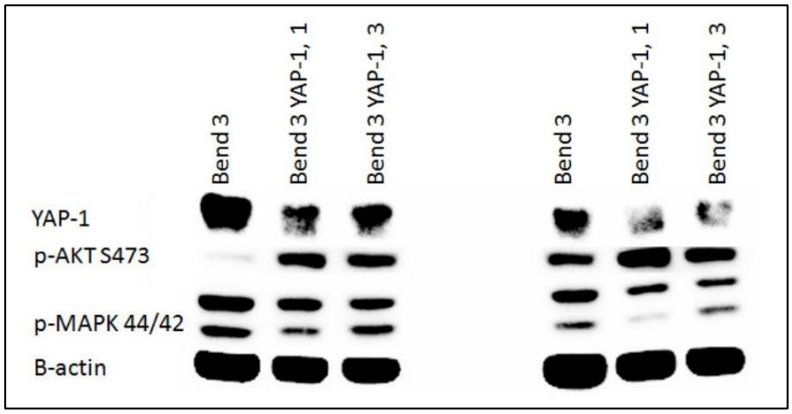

Figure 2: Western Blot of YAP-1 and other relevant proteins in BEND3 control (left) and treatment (right) cells.

Discussion

The successful knockdown of the YAP-1 protein in the BEND3 YAP-1,1 and BEND3 YAP-1, 3 cells show that there is potential for a novel therapy to be developed using this siRNA technique. The YAP-1 protein, which has been identified as an oncogene and an upregulated factor in tumors such as infantile hemangioma, could have mediating properties when expressed at normal levels similar to that in healthy tissue. The successful knockdown of this protein is the first step in

developing a therapy that uses an siRNA technique to potentially mediate tumor growth of infantile hemangioma.

Additionally, other relevant proteins were affected by the siRNA technique as observed by the western blot. P-MAPK 44/42, a protein associated with the development of cancers, appears to be down regulated in the BEND3 YAP-1,1 and BEND3 YAP-1,3 cells. Additionally, p-AKT S473, a protein used as a pathway for proliferation and cell survival, appears to be upregulated in the BEND3 YAP-1,1 and BEND3 YAP-1,3 cells. The impact of the YAP-1 knockdown on these proteins suggests that further research must be conducted to determine the effect of these varied protein levels on tumor prognosis as this treatment is further developed

Recently, cancer research has increasingly focused on investigating targeted therapies in attempts to discover novel treatments for various cancer. Targeted therapies, as defined by the National Cancer Institute, act on specific molecular targets that are associated with cancer, whereas most standard chemotherapies act on all rapidly dividing normal and cancerous cells. [8] Because of their specificity, targeted therapies are often less-invasive and more successful in suppressing cancer growth and formation. It is useful to investigate targeted therapies in treating benign tumors as well as cancerous tumors.

The results of this study lay the foundation for investigating targeted therapies for infantile hemangioma. Targeting YAP-1 in hemangioma cells could have a suppressing effect on the proliferation and vascularization of the tumor. It is prudent that this targeted therapy as well as others are explored as potential alternatives to current treatments available for infantile hemangioma. A new treatment is needed and would alleviate the maladies and dangerous risk factors associated with current infantile hemangioma treatments that are administered orally, topically, and intravenously.

Additionally, the results of the western blotting reveals that the presence of the shYAP-1 gene effects multiple proteins in addition to YAP-1. These results are relevant because it shows that when one protein is targeted, other pathways and proteins relevant to tumor development can also be affected. When a therapy is developed, it is important to understand all the effects that it has on signaling pathways so that the true effects of the drug can be predicted. Examining a wide range of signaling pathways and proteins will be critical as targeted therapies, including a YAP-1 knockdown, are further investigated.

References

1. Chen, T., Eichenfield, L., & Friedlander, S. (2013). Infantile Hemangiomas: An Update on Pathogenesis and Therapy. *Pediatrics, 131*(1), 99-108.

2. Chik K., Luk C., Chan H., Tan H (2010). Use of propranolol in infantile haemangioma among Chinese children. *Hong Kong Med J*. 16,341–346.

3. George, M. (2004). Adverse Effects of Systemic Glucocorticosteroid Therapy in Infants With Hemangiomas. *Archives of Dermatology, 140*(8), 963-969.

4. Ji, Y., Chen, S., Li, K., Li, L., Xu, C., & Xiang, B. (2014). Signaling pathways in the development of infantile hemangioma. *Journal of Hematology & Oncology, 7*(13).

5. Oksiuta, M., Matuszczak, E., Dębek, W., Dzienis-Koronkiewicz, E., Hermanowicz, A., & Tylicka, M. (2014). Treatment of problematic infantile hemangiomas with propranolol: A series of 40 cases and review of the literature. *Progress of Hygiene and Experimental Health,* (68), 1138-1144.

6. Sethi, S., Li, Y., & Sarkar, F. (2013). Regulating miRNA by Natural Agents as a New Strategy for Cancer Treatment. *Current Drug Targets, 14*(10), 1167-1174.

7. Shehata N, Powell J, Dubois J, Hatami A, Rousseau E, Ondrejchak S, McCuaig C (2013): Late rebound of infantile hemangioma after cessation of oral propranolol. *Pediatric Dermatology*, 30(5):587–591

8. Targeted Cancer Therapies. (2014, April 25). Retrieved April 13, 2015, from http://www.cancer.gov/cancertopics/treatment/types/targeted-therapies/targeted-therapies-fact-sheet

9. Wang, H., Du, Y., Zhou, X., Liu, H., & Tang, S. (2014). The dual functions of YAP-1 to promote and inhibit cell growth in human malignancy. *Cancer Metastasis Review, 33*(1), 173-81.

10. Wang, W., Huang, J., & Chen, J. (2011). Angiomotin-like Proteins Associate with and Negatively Regulate YAP1. *Journal of Biological Chemistry, 286*(6), 4364-4370.

11. Zhang, Z., Men, T., Feng, R., Li, Y., Zhou, D., & Teng, C. (2013). MiR-375 Inhibits Proliferation of Mouse Pancreatic Progenitor Cells by Targeting YAP1. *Cellular Physiology and Biochemistry, 32*(6), 1808-1817.